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Induction of Apoptosis in HT-29 Cells Infected With SA-11 Rotavirus

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Rotavirus infection is associated both in vivo and in vitro with a series of subcellular pathological alterations leading to cell lysis. It has been suggested that these modifications can play a key role in the pathogenesis of rotavirus-associated diarrheal disease. We describe the effects of SA-11 rotavirus infection in HT-29 cells, a human enterocyte-like cell line. Cytological analyses suggested that the viral-induced cytopathic process, including chromatin clumping, can be referred to as apoptosis, the cell death pathway alternative to necrosis. A time course of the process was performed to investigate whether rotavirus-associated cell death showed specific injury signs. HT-29-infected cells were analyzed by scanning and transmission electron microscopy and features of apoptosis such as blebbing of the plasma membrane, peripheral condensation of chromatin, and fragmentation of the nucleus were observed. Specific changes occurring in cell-substrate adhesion and in some organelles relevant for viral maturation, i.e., rough endoplasmic reticulum, were detected. These findings indicate a role for apoptosis in the rotavirus infection process and its related cytopathology, and also suggested that specific histological alterations such as derangement of enterocytes are associated with the pathogenesis of rotavirus-induced diarrheal disease and could be a direct consequence of viral-triggered apoptosis.
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KEY WORDS: Apoptosis, rotavirus, in vitro

INTRODUCTION

Rotaviruses are nonenveloped double-stranded RNA viruses that belong to the Reoviridae family. They have been identified as the most common cause of symptomatic gastroenteritis in infants and young children in both developed and developing countries [Kapikian and Chanock, 1996]. These viruses have also been involved in outbreaks of diarrhea occurring in nursing homes, among travelers, in day-care centers, and adult contacts of ill children. Rotavirus infection is characterized by specific cell and tissue tropism. The virus has been shown to attach to and infect mature enterocytes lining the upper portions of the villi of the small intestine [Blacklow and Greenberg, 1991]. This results in cell lysis and stripping of the superficial layer of epithelial cells from the underlying lamina propria.

Programmed cell death, or apoptosis, is defined as a physiological cell suicide process alternative to necrosis and is morphologically characterized, in vivo, by cell-to-cell contact derangement, DNA aggregation and fragmentation, rapid shrinking, and budding of the cell with the formation of membrane-enclosed apoptotic bodies and blebs containing well-preserved organelles [Wyllie, 1981; Kerr et al., 1984; Wyllie et al., 1984; Schwartzman and Cidlowski, 1993]. It has been hypothesized that apoptosis may play a central role in embryonic development, such as thymocyte selection, as well as in human malignancies [Evans, 1993] and in the pathogenesis of several infectious diseases [Zychlinsky, 1993]. The apoptotic process can be experimentally induced in vitro by using certain chemical and physical agents such as drugs or cytokines. In addition, more recently, studies investigating the virus-associated lytic processes have been carried out and an important role for apoptotic cell death pathway in numerous viral infections has been found [Groux et al., 1991; Cohen, 1991; Ameisen and Capron, 1991; Hugin et al., 1991; Morey et al., 1993; Yamada et al., 1994; Hinshaw et al., 1994; Vasconcelos and Lam, 1994; Esolen et al., 1995]. In particular, different viruses belonging to various families have been demonstrated to induce, or to prevent, apoptosis [McCabe and Orrenius, 1992; Meyaard et al., 1992; Rojko et al., 1992; Jeurissen et al., 1992; Thompson, 1995]. However, general rules for viral infection-related cell death have still to be established. The present work focuses on the possible relation between

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rotavirus infection and apoptosis, with the aim to gain further insights into the cellular changes occurring in rotavirus-infected intestinal cells. We have exploited the in vitro model of HT-29 cells, an enterocyte-like cell line which has been shown by our group to be highly permissive to viral replication [Superti et al., 1991]. We report results indicating a role of apoptosis in the rotavirus-associated cytopathology.

MATERIALS AND METHODS

Cells

The human colon adenocarcinoma cell line HT-29 was obtained from the American Type Culture Collection (ATCC; Rockville, MA). It was grown at 37°C in RPMI 1640 medium (Gibco) containing 1.2 g/l $NaHCO_3$ and supplemented with 10% inactivated fetal calf serum (FCS; Flow Laboratories), 2 mM glutamine, nonessential amino acids, penicillin (100 IU/ml), and streptomycin (100 μg/ml).

Virus

Simian rotavirus SA-11 was grown in LLC-MK_2 cells (a monkey kidney cell line). The virus was preactivated with 20 μg/ml trypsin type IX (Sigma Chemical Company) for 30 min at 37°C, diluted 10-fold in 199 medium (HyClone), and then inoculated onto a confluent cell monolayer grown in roller bottles at a multiplicity of infection (m.o.i.) of 5 plaque forming units (p.f.u.)/cell. After 90 min at 37°C, the inoculum was removed, and the monolayers were washed once with phosphate-buffered saline (PBS; pH 7.4) and then incubated at 37°C in 199 medium containing 1 μg/ml trypsin. When extensive cytopathic effect was observed, infected cultures were frozen and thawed three times, centrifuged (3,000*g* for 10 min), and supernatants were stored at −70°C.

This stock virus was titered by plaque assay on LLC-MK_2 cells utilizing an overlay medium containing 0.6% purified agar, 3 μg/ml trypsin, and 50 μg/ml DEAE dextran (all from Sigma Chemical Company).

Subcellular Analysis of Infected Cells

HT-29 cells, grown for 48 hr at 37°C, were infected with preactivated SA-11 rotavirus (m.o.i. of 1 and 10 p.f.u./cell). After 1 hr adsorption at 37°C, the cells were washed with RPMI 1640 medium to remove the inoculum and incubated at 37°C for different times (8, 16, and 24 hr). DNA fragmentation, cell necrosis, and cell viability were determined by acridine orange-ethidium bromide staining. Apoptosis was also evaluated by Hoechst 33258 dye analysis.

All experiments were carried out on virus-infected and "mock-infected" cells. Mock-infected cells are control cells incubated with trypsin-treated RPMI 1640 medium for 1 hr at 37°C, washed, and then incubated at 37°C for the same intervals of time as described for viral infection.

Acridine Orange-Ethidium Bromide Staining

Mock-infected and virus-infected cells were stained with an acridine orange (100 μg/ml)-ethidium bromide (100 μg/ml) solution. Ten microliters of the stained cell suspension, placed on a glass microscope slide and covered with a 22 mm^2 coverslip, was examined under u.v. illumination (Nikon). In all experiments each sample was analyzed in triplicate by counting at least 200 or more cells for replicate. The characteristics of the cells were recorded according to the color and the structure of the chromatin [Duke and Cohen, 1992].

Hoechst 33258 Staining

In order to evaluate apoptotic cell death, virus-infected and mock-infected cells were washed with PBS and fixed with 3.7% formaldehyde in PBS for 10 min at room temperature. After washing in the same buffer, the cells were permeabilized with 0.5% Triton X-100 (Sigma Chemical Company) in PBS for 10 min at room temperature. The chromatin dye Hoechst 33258 was used to visualize clumps of DNA [Bursch et al., 1992; Malorni et al., 1993]. After washing, all samples were mounted in buffered glycerol and viewed using a Nikon Microphot fluorescence microscope.

Detection of Apoptosis by Propidium Iodide Staining and Flow Cytometry

In order to evaluate apoptosis after 8, 16, and 24 hr of infection with 10 p.f.u./cell, mock-infected and virus-infected cells were harvested and pooled with the supernatants. The cell suspension from each sample was fixed in 80% ethanol at +4°C for 60 min. Cells were then washed twice in PBS, centrifuged, and resuspended in a solution containing 50 μg/ml (50 U/ml) RNase and 50 μg/ml propidium iodide (PI). Samples were analyzed in a FACScan cytofluorimeter. The logarithmic red PI fluorescence was measured with an LP 620 filter. Twenty thousand events per sample were accumulated and the list mode data analyzed by Lysis II-C32 Becton Dickinson software.

Scanning Electron Microscopy (SEM)

Control and rotavirus-infected cells (5 p.f.u./cell) were processed for SEM as described previously [Malorni et al., 1994]. Briefly, cells were seeded on glass coverslips and after 8, 16, and 24 hr were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) containing 2% sucrose (w/v) at room temperature for 20 min. Following postfixation in 1% osmium tetroxide for 30 min, cells were dehydrated through graded ethanols, critical point dried in CO_2, and gold coated by sputtering. Observations were made with a Cambridge 360 scanning electron microscope.

Transmission Electron Microscopy (TEM)

HT-29 cells infected with preactivated SA-11 rotavirus (5 p.f.u./cell) and control cells were processed for TEM. At different times postinfection (8, 16, and 24 hr) cells were harvested, washed in PBS, and fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) at

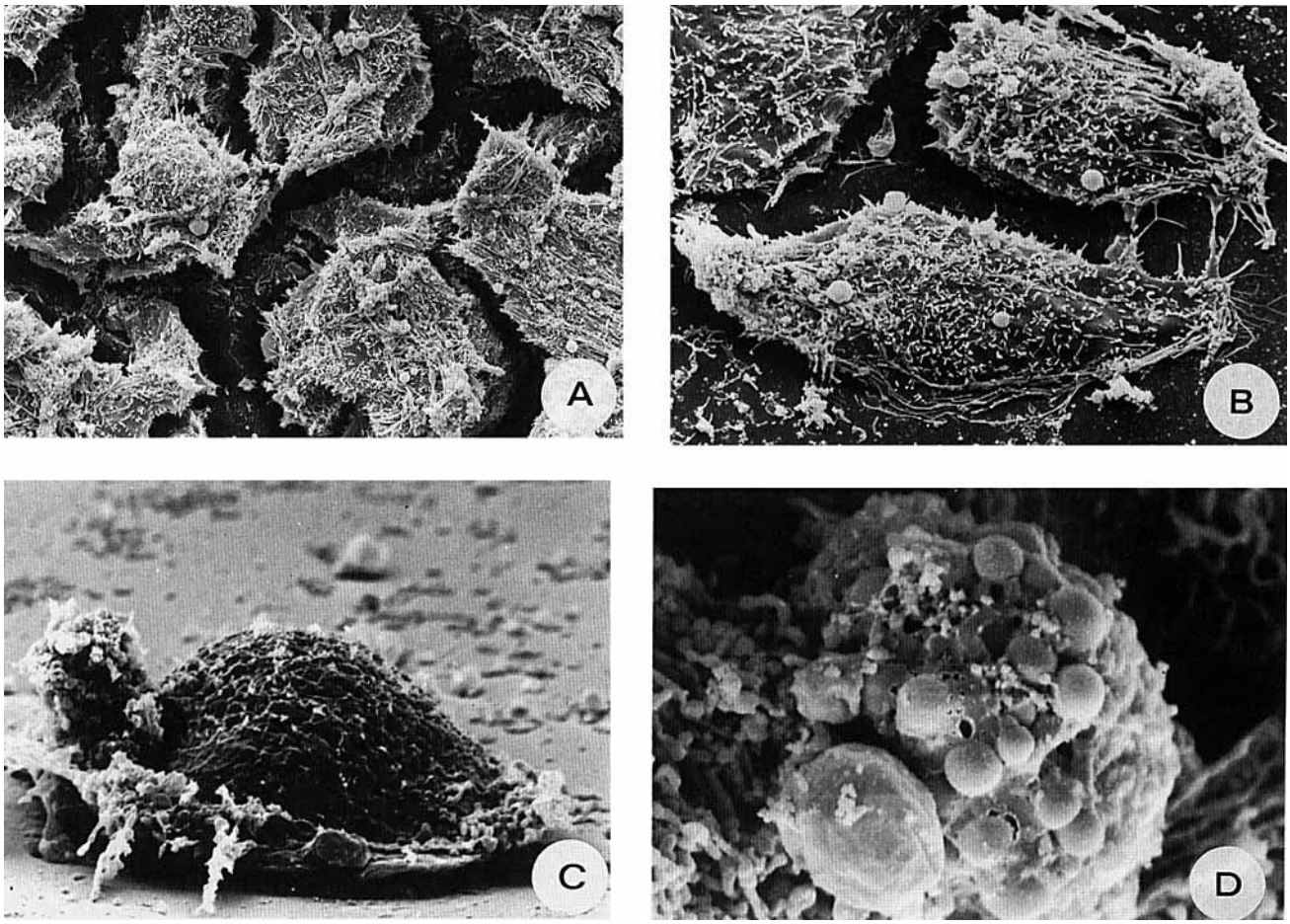


Fig. 1. SEM. HT-29 cell surface, as seen by SEM analysis, is mainly characterized by numerous microvillous structures and ruffles **(A)** which appear to be markedly altered after 8 hr of viral exposure **(B)**. These changes lead to cell retraction and loss of the cell-substrate relationships after 16 hr from rotavirus inoculum **(C)**. As a final event 24 hr postinfection **(D)**, cell rounding and shrinking with formation of surface protrusions such as blebs and blisters are observed. ×1,200 (A), ×1,800 (B), ×3,400 (C), and ×8,600 (D).

room temperature for 30 min. Cells were then postfixed in 1% osmiun tetroxide in cacodylate buffer, dehydrated through graded series of ethanol solutions and, finally, embedded in Agar 100 epoxy resin. Thin sections were stained with lead citrate and uranyl acetate and examined with a Zeiss 10 C electron microscope.

RESULTS

Cytological Analyses

The effects of viral infection were evaluated in HT-29 cells by means of different approaches in order to analyze 1) the time course of the cytopathic process leading to cell injury and 2) the final result of such alterations, i.e., necrotic or apoptotic cell death. The role of different viral concentrations in the occurrence of the above cited phenomena was also considered.

When observed by means of brightfield light microscopy, HT-29-infected cells were seen to undergo a number of morphological changes with respect to control cells. In particular, uninfected cells appeared to grow as cell clusters and in patches, while the progression of infection induced cells to round up detaching from each other and from the substrate, finally floating in the medium (data not shown). These alterations were better characterized by SEM analysis which showed surface alterations of HT-29 cells occurring at 8, 16, and 24 hr postinfection (Fig. 1). Cell surface changes were well visible after 8 hr and were mainly represented by alterations of the microvillous structure (Fig. 1B) which appeared to be markedly modified as compared to control cells (Fig. 1A). These changes lead to the loss of cell-to-cell and cell-substrate contacts (Fig. 1C), followed by rounding and blebbing (Fig. 1D), eventually leading to cell detachment from the substrate.

Cell Death Analyses

The percentage of apoptotic, necrotic, and viable cells at the different times of infection is shown in Figure 2. Typical apoptotic cells increased as the time of infection went on. As a matter of fact, at 8, 16, and 24 hr postinfection with a m.o.i. of 1, the percentage of apoptotic cells was 7.5%, 27.5%, and 35%, respectively (Fig. 2a). The

highest levels of apoptosis were observed with an m.o.i. of 10 which, in the same time intervals, induced apoptotic death in 10%, 30%, and 65% of the cells, respectively. The percentage of apoptotic cells in negative controls at 8, 16, and 24 hr did not exceed 5%, 7.5%, and 9.5%, respectively. Hence, a relationship between apoptosis and virus concentration seemed to occur so that at 16 and 24 hr postinfection, cells infected with an m.o.i. of 1 or 10 displayed statistically significant differences in the apoptotic indices as compared to mock-infected cells ($P < 0.0001$). In some experiments, 24 hr after infection with an m.o.i. of 10, the assay was separately performed on attached and detached cells. In floating cells, the chromatin alterations appeared more frequently than in substrate-adhering cells (data not shown). Concerning necrosis induction, its incidence did not increase as much as apoptosis (Fig. 2b), reaching the percentage of 21.5% after 24 hr of infection with 10 p.f.u./cell. Results obtained on cell viability are shown in Figure 2c. After 8 and 16 hr, only slight differences were observed between the negative controls and infected cells. After 24 hr, the percentage of viable infected cells was 65% and the percentage of viability in mock-infected cells was 85%. In these experiments, additional controls were included by utilizing virus inactivated according to Offit and Dudzik [1989]. Briefly, stock virus was mixed with β-propiolactone (0.15%), maintained at 4°C for 72 hr, and then at 37°C for 3 hr. This inactivated virus was added to the cells in the same experimental conditions utilized for viral infection. The apoptotic rates induced by β-propiolactone-treated virus were quite similar to those observed in uninfected cells as, 24 hr postinfection with an m.o.i. of 10, apoptosis was observed in about 10% of cells (data not shown).

A qualitative analysis of apoptotic figures was also carried out (Figs. 3, 4). In the experimental conditions described above, rotavirus infection induced specific signs of apoptosis (Fig. 3A). Some features of apoptosis were observed at 8 hr postinfection (Fig. 3B) and typical apoptotic nuclei, characterized by chromatin aggregation or by clumping with several necrotic debris, were observed after 16 hr (Fig. 3C). The amount of cells displaying the apoptotic morphology increased with the progression of infection. In fact, 24 hr postinfection, apoptotic figures were well evident in a large part of the cell population (Fig. 3D). In addition, specific rotavirus-associated morphologies were found, in which aggregation and condensation of chromatin were followed by typical clumping. The main features of these cells were represented by a characteristic chromatin aggregation (Fig. 4A) which could precede other alterations such as shrinking of this modified structure (Fig. 4B,C), possibly leading to the small, single (Fig. 4D,E) or the multiple and extended (Fig. 4F) chromatin clumps.

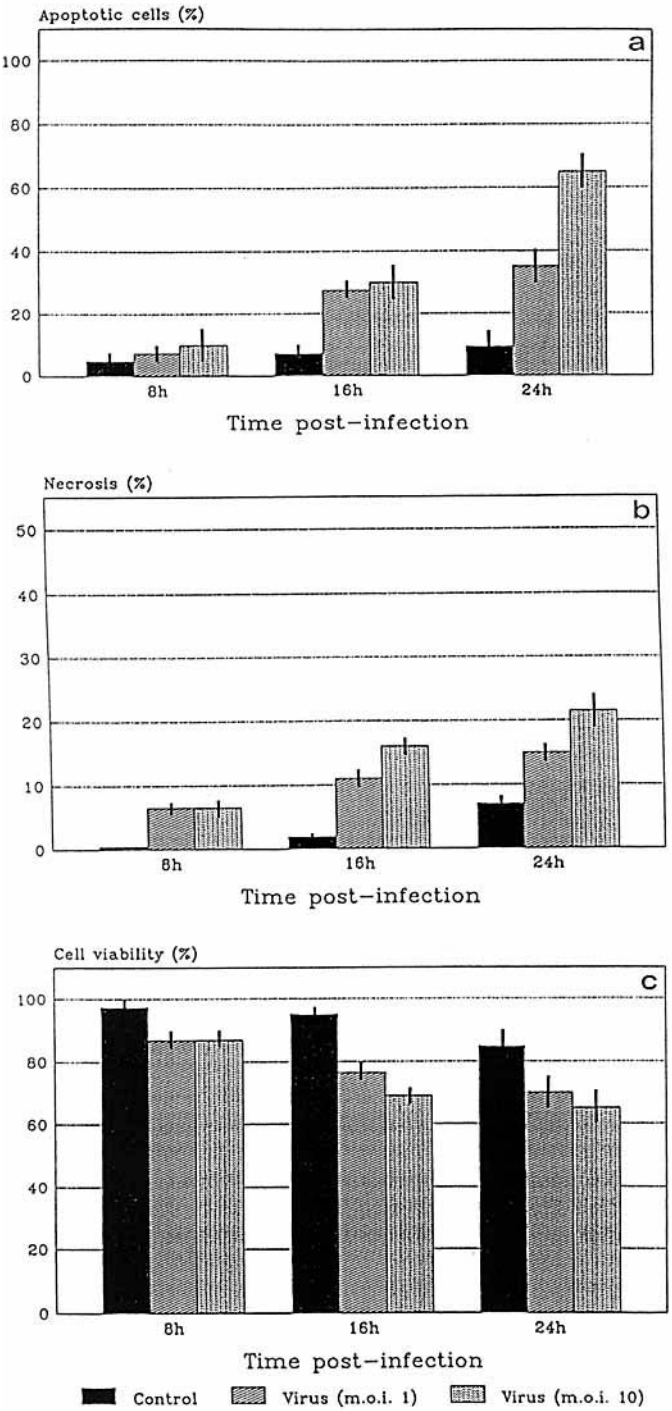


Fig. 2. Percent of apoptosis **(a)**, cell necrosis **(b)**, and cell viability **(c)** in HT-29 cells infected with rotavirus (m.o.i. of 1 and 10; 8, 16, and 24 hr postinfection).

Detection of Apoptosis by PI Staining and Flow Cytometry

The percentage of apoptotic cells at different times after infection with 10 p.f.u./cell was also determined by flow cytometric analysis. Results obtained by PI staining were quite similar to those obtained with other intercalating DNA dyes (such as acridine orange and ethidium bromide), or dyes binding externally to DNA (such as Hoechst 33258). In fact, as shown in Figure 5, the infection of HT-29 cells with an m.o.i. of 10 for 24 hr induced the apoptotic process in a significant proportion of the cell population (60%).

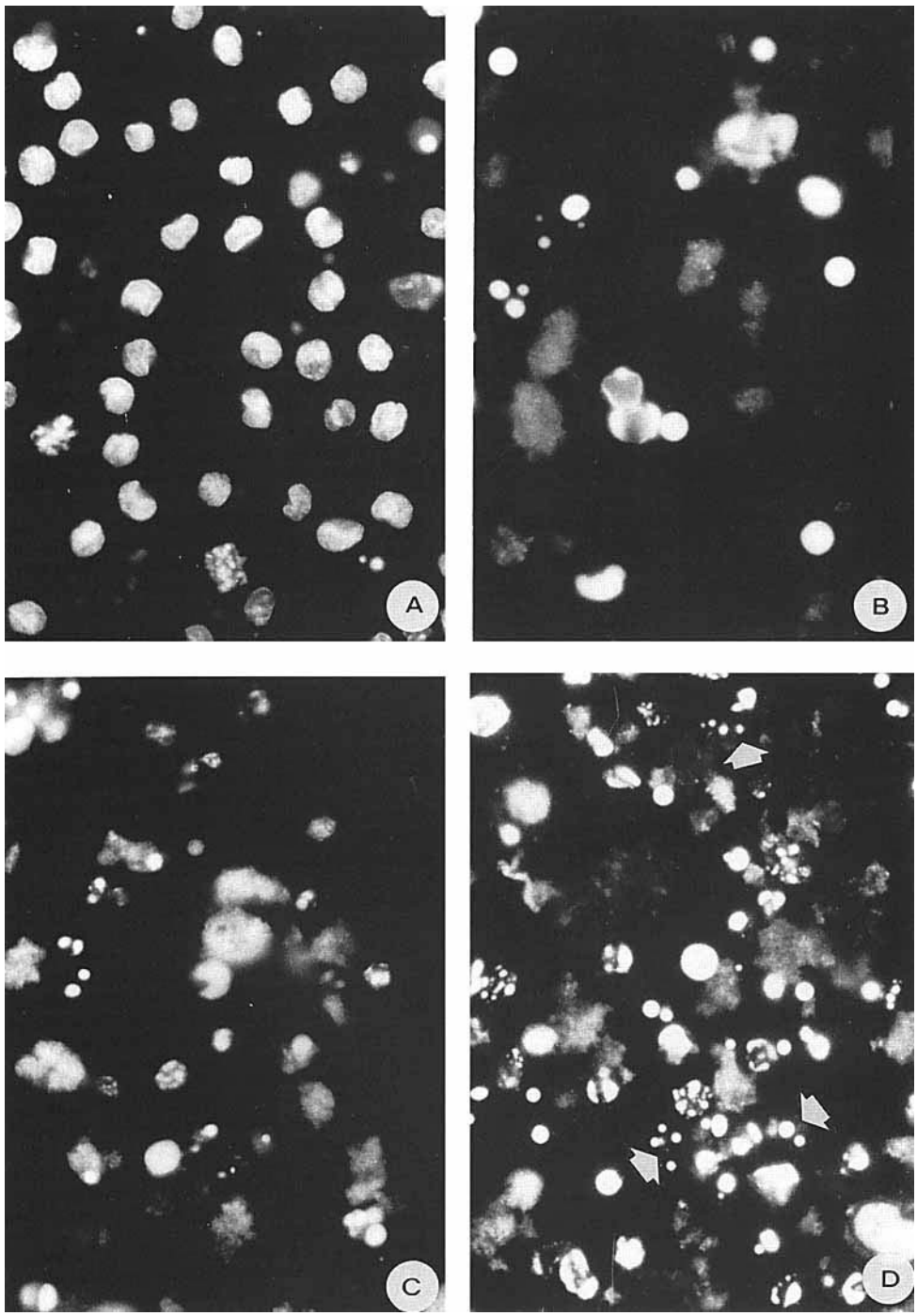


Fig. 3. Apoptotic features. Cell population is represented, in control samples, by normally shaped chromatin figures with some isolated mitoses **(A).** After 8 hr of rotavirus exposure, i.e., when viral replication is still in progress, only the occurrence of a few signs of cell injury, mainly represented by the appearance of cell debris, is detectable **(B).** In contrast, after 16 hr **(C)** and even more after 24 hr **(D),** i.e., once the viral progeny formation is completed, numerous typical apoptotic figures are detectable (arrows). ×215 (A, C, D); ×430 (B).

Time Course Ultrastructural Features

To substantiate further the occurrence of apoptosis in rotavirus infection, mock-infected and virus-infected HT-29 cells were analyzed in their ultrastructural features. Uninfected control cells and rotavirus-infected cells (8 hr postinfection) are shown in Figure 6. When compared to control cells (Fig. 6A), HT-29-infected cells displayed some morphological changes (Fig. 6B). In particular, chromatin rearrangement at the nuclear periphery together with numerous viral particles, enclosed within rough endoplasmic reticulum cisternae, was observed. In addition, some virions were also detected between the outer and the inner perinuclear membrane (Fig. 6B, inset). Although slightly modified, no signs of damage were detected at this time of infection in the cytoplasmic organelles. A specific series of experiments at different time intervals (8, 16, and 24 hr postinfection) was then undertaken in order to verify whether the time course of the infection was paralleled with specific apoptotic stages. Careful ultrastructural

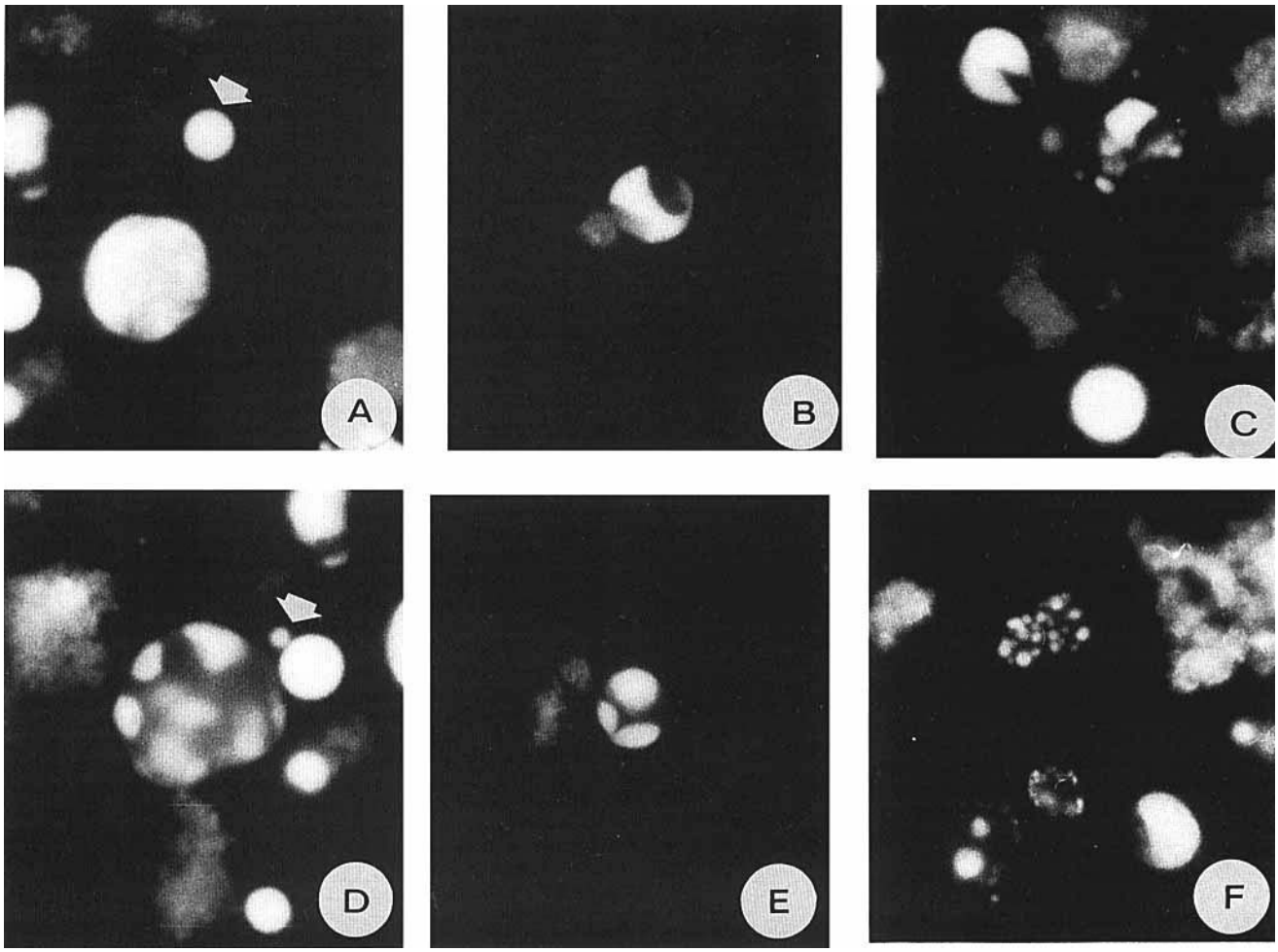


Fig. 4. Different apoptotic morphologies at 24 hr postinfection. Chromatin condensation (**A,** arrow) is paralleled to other aggregation figures showing typical shrinking and initial clumping **(B, C).** A single nuclear bleb (**D,** arrow) as well as typical clumping and fragmentation (A, C, D, E, F) are evident. ×625 (A, C, D); ×312.5 (B, E, F).

examination of both adherent and detached cells showed more advanced changes in floating cells. The progressive evolution of the programmed cell death process was found as shown in Figure 7 and could be divided in at least four different morphological stages. An early phase at 8 hr postinfection was mainly represented by i) slight changes in the density and distribution of nuclear chromatin that condensed peripherally into a crescent-shaped mass, ii) a loss of cell-to-cell relationships, and iii) the detection of numerous viral particles in the cell cytoplasm (Fig. 7A). At 16 hr postinfection, it was more difficult to detect viral particles which were mostly localized in the perinuclear cisternae. At this time it was possible to distinguish a second and a third phase of the apoptotic process. In the second morphological stage (Fig. 7B,C), cells were detached both from the substrate and from the neighboring cells with the nucleus exhibiting several large peripheral chromatin clumps. Morphological signs of cell injury, such as surface blebbing (Fig. 7B) and intracytoplasmic vacuolation (Fig. 7C), were also detected. Furthermore, in this phase, nuclear pores appeared to be dilated and clustered in certain nuclear regions (Fig. 7C). In a third phase, alterations progressed in a wide dilation of intracytoplasmic vesicles and in a derangement of chromatin structure (Fig. 7D). In some areas, it was also possible to observe a few viral particles segregated in the perinuclear cisternae (Fig. 7E, inset). Finally, in a fourth properly lytic phase, 24 hr after infection, cells displayed the typical markers of apoptotic cell death such as nuclear segmentation, cytoplasmic derangement and disruption, and release of viral particles (Fig. 7F).

DISCUSSION

Although clinical pathological studies have established clearly that the cellular target of rotavirus is the differentiated enterocyte of the small intestine, no in vitro studies aimed to evaluate the cellular changes occurring after viral replication in intestinal cells have been reported. This reflects in part the difficulties in

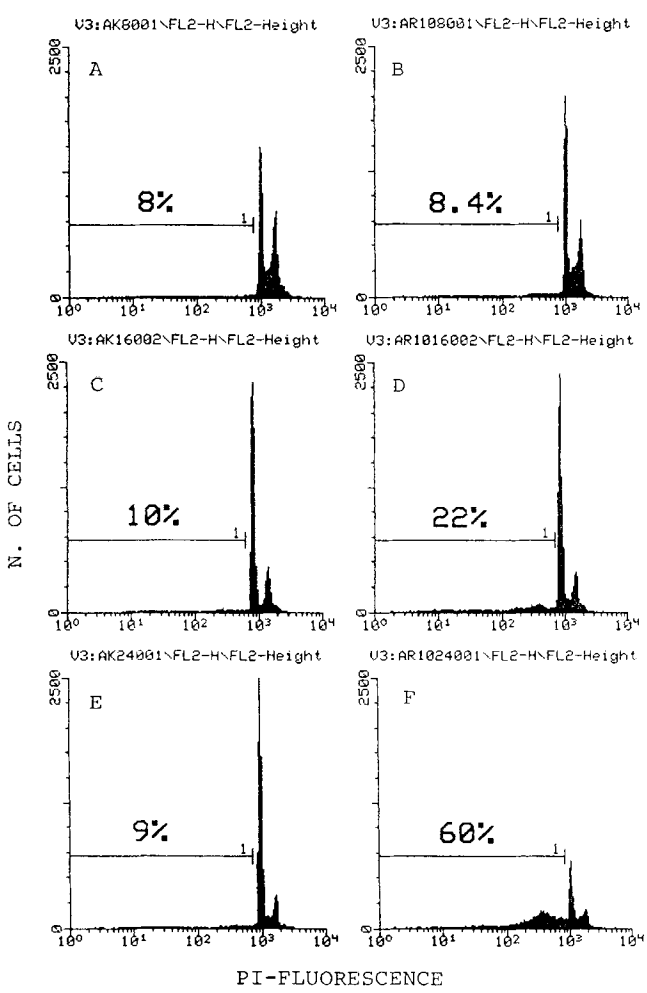


Fig. 5. **A, C, E:** DNA histograms of PI-stained mock-infected cells and **B, D, F:** virus-infected cells (m.o.i. of 10) at different times postinfection (8, 16, and 24 hr, respectively).

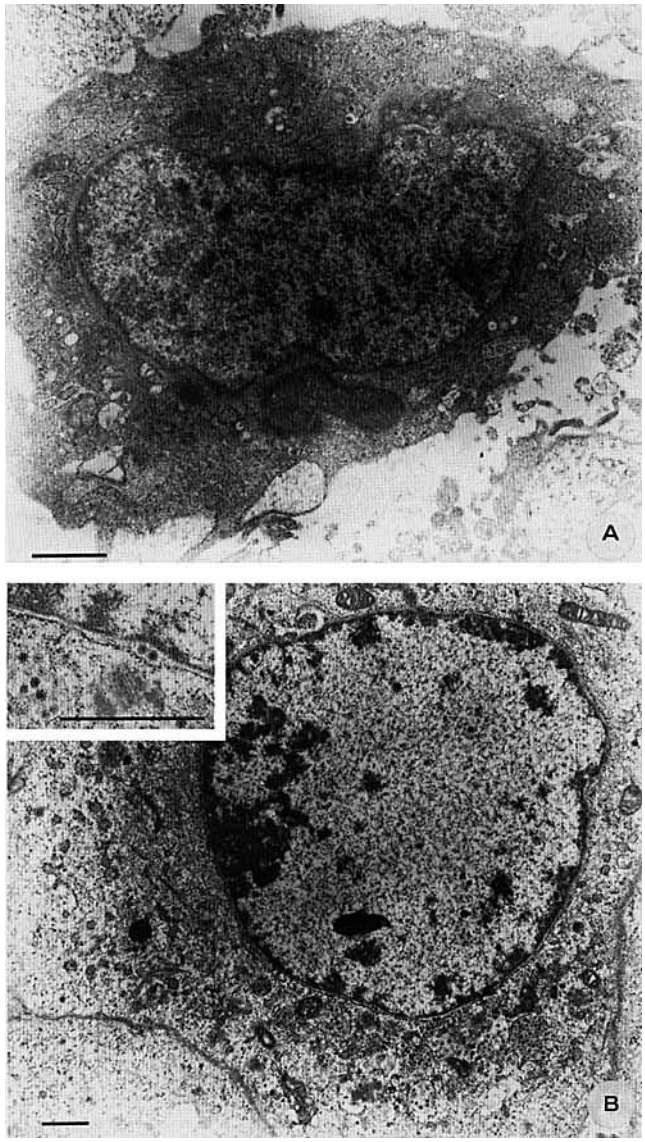
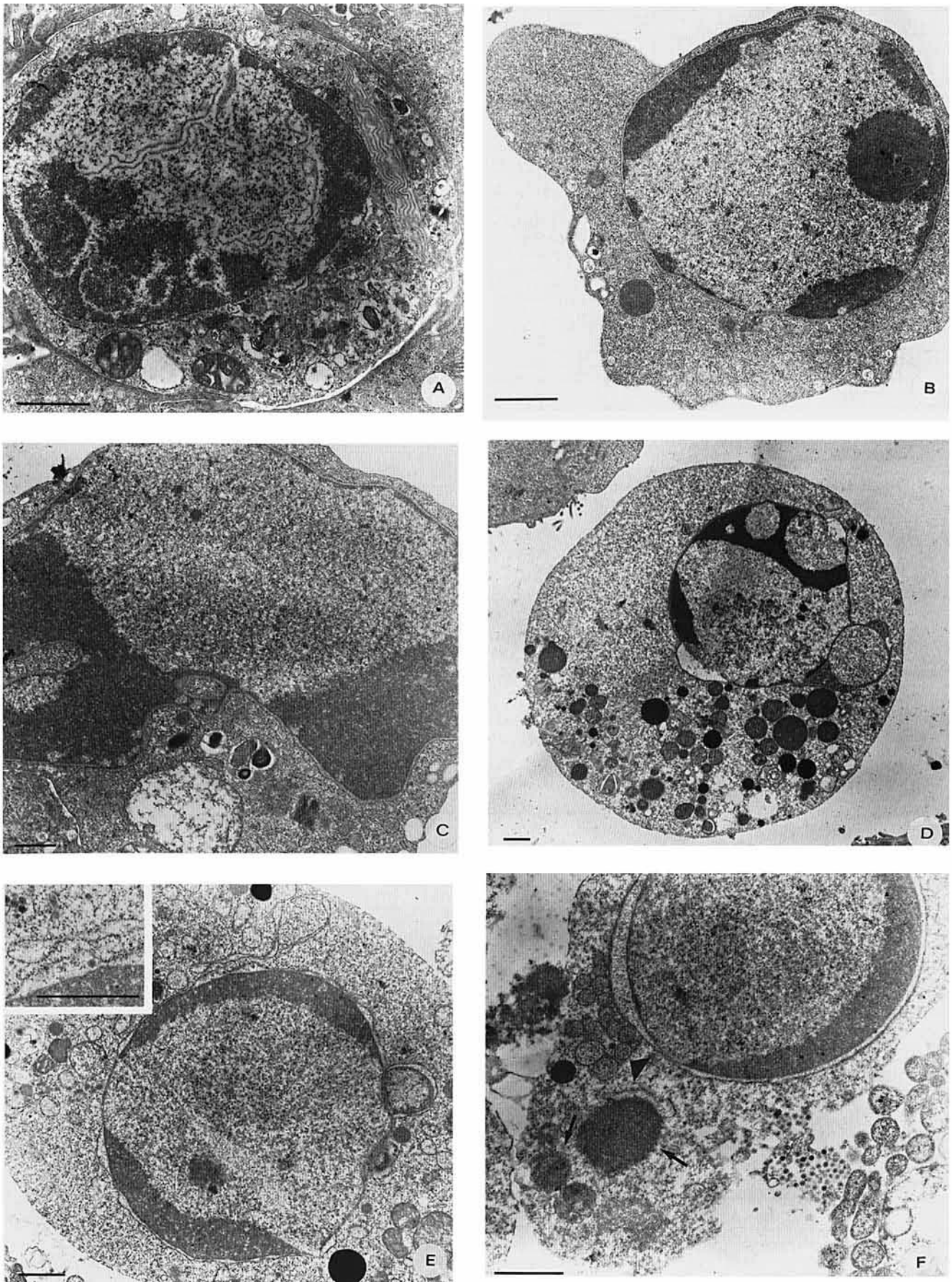


Fig. 6. Electron micrographs showing **(A)** mock-infected HT-29 cells and **(B)** rotavirus-infected cells (8 hr postinfection). The nucleus undergoes slight changes while cytoplasmic organelles are still well preserved. Note virus particles within the perinuclear membrane (inset). Bars represents 1 μm.

culturing differentiated intestinal cells, as the few cell lines derived from the small intestinal mucosa did not exhibit the morphological features of differentiated enterocytes [Quaroni and May, 1980; Quaroni, 1985]. Such a limitation has been surmounted recently by the demonstration of the high susceptibility to rotavirus infection of the long-term cell line HT-29 [Superti et al., 1991]. In fact, in spite of their colonic derivation, this cell line mimics the situation found in the small intestine, being able to express differentiation features which are characteristic of mature intestinal cells [Rousset, 1986]. HT-29 cells may therefore represent a valuable in vitro tool for a better understanding of the rotavirus-intestinal cell interactions.

This experimental model was used to study the effect of rotavirus replication and the data obtained suggest a role for programmed cell death process in the virus-associated cytopathology.

Apoptosis is a morphological and biochemical process, distinct from cell necrosis, which has been increasingly recognized to play a relevant role in a variety of biological phenomena such as metamorphosis, embryogenesis, cell differentiation, and tumor regression [Wyllie, 1981; Smith et al., 1989]. This mechanism of cell death has also been reported following infection by several viruses. Indeed, viruses inducing apoptosis as well as viruses inhibiting the apoptotic process have been described [Thompson, 1995]. For instance, apoptosis has been associated with infection by human hepatitis B virus [Desmet, 1988], herpes virus [Tropea et al., 1995], influenza virus [Hinshaw et al., 1994], measles virus [Esolen et al., 1995], parvovirus B19 [Morey et al., 1993], infectious bursal disease virus [Vasconcelos and Lam, 1994], feline leukemia virus [Rojko et al., 1992], and human immunodeficiency virus [Ameisen and Capron, 1991; Meyaard et al., 1992].


A
B
C
D
E
F

Results of our studies by acridine orange-ethidium bromide staining showed a close relationship between apoptosis and viral replication and also indicated apoptosis as a virus concentration-dependent process. With the progression of infection, the apoptotic indices became increasingly higher than the necrotic ones. Thus, also it has been assumed previously that rotavirus infection results in cell death mainly by a lytic process [Estes, 1996], the present findings suggest that death of rotavirus-infected intestinal cells may result not only by necrosis but also, and mostly, through the activation of programmed cell death.

In spite of the detection of chromatin fragmentation by either DNA intercalating fluorescent dyes or dyes binding externally to DNA, we were unable to reveal the DNA ladder by agarose gel electrophoresis (data not shown). The results are in agreement with those reported by Bertrand et al. [1991] who failed to detect a DNA ladder in apoptotic HT-29 cells treated with teniposide. It is likely that, as suggested by Bertrand et al. [1991], in HT-29 cells topoisomerase II-mediated DNA breaks reverse rapidly and completely, so impairing the detection of internucleosomal DNA fragmentation by endonucleolitic cleavage.

Moreover, the ability to observe morphological features of apoptosis together with the inability to demonstrate simultaneous internucleosomal cleavage of DNA to 180 bp integers concurs with the observations of other investigators [Cohen et al., 1992; Oberhammer et al., 1993]. In particular, results from studies of Oberhammer et al. [1993] carried out on different cell lines showed that, despite the appearance of condensed chromatin in each cell type, not all cell types underwent internucleosomal cleavage to generate 180 bp integers. This suggests that the appearance of a DNA ladder is not mandatory in an apoptotic cell death defined by characteristic changes in chromatin condensation.

Our results from FACScan analysis by PI staining of infected cells further indicate that rotavirus infection induces chromosomal margination and separation. In the late stages of infection, 60% of infected cells displayed DNA degradation. Ultrastructural analysis by TEM confirms the results obtained by morphological assays with fluorescent dyes demonstrating that peculiar features can be found in HT-29 cells undergoing apoptosis. Morever, when ultrastructural analysis was carried out on both adherent and detached cells, more advanced morphological apoptotic alterations were observed in floating cells. The data are in agreement with the results obtained by others [Bertrand et al., 1991; Desjardins and MacManus, 1995], showing that HT-29 cells treated with teniposide undergo typical apoptosis displaying different stages of programmed cell death depending on the cell-substrate relationships. The importance of such relationships in apoptosis triggering was also suggested for other experimental conditions [Rouslahti and Reed, 1994; Malorni et al., 1995]. Moreover, by electron microscopy examination viral particles, mostly localized within the rough endoplasmic reticulum cisternae and the dilated perinuclear double membrane, were more easily detected at an early apoptotic phase than toward the end of the process leading to cell death, when mature virions were released from the cells.

It has been reported previously that plasma membrane calcium permeability may increase as a result of rotavirus infection depending on the cumulative viral protein synthesis during early times of infection [Michelangeli et al., 1991]. Thus, the cytopathic effect of rotavirus on host cells appears to be mediated by an increase in intracellular Ca^{++} induced by the synthesis of a viral product. It has also been reported that reduction of extracellular Ca^{++} concentration affected the rotavirus cytopathic effect [Shahrabadi et al., 1987]. In particular, rotavirus maturation requires high Ca^{++} concentration in the cisternae of the endoplasmic reticulum, while virus-mediated cytopathic effect depends on an elevated Ca^{++} concentration in the cytosol [Michelangeli et al., 1995]. As a matter of fact, it has been demonstrated recently that the nonstructural glycoprotein NSP4 alters endoplasmic reticulum Ca^{++} permeability [Tian et al., 1995]. As disturbances in intracellular Ca^{++} homeostasis are linked to the onset of programmed cell death in several cell systems [Nicotera et al., 1992], it is also likely that apoptosis induced by rotavirus infection could be a Ca^{++}-mediated process. The mechanism by which viral infection increases intracellular Ca^{++} concentration is similar to that observed after cell treatment with the microsomal Ca^{++}-ATPase inhibitor thapsigargin, which is itself sufficient to activate DNA fragmentation [Zhivotovsky et al., 1994]. Further studies are needed on the role of calcium in rotavirus infection as our data suggest that not only necrosis but also apoptosis should be considered in the study of the cell lytic process by rotavirus.

Fig. 7. Progressive evolution of programmed cell death in rotavirus-infected HT-29 cells. At 8 hr postinfection, a crescent-shaped mass of nuclear chromatin together with a loss of cell-to-cell relationships can be observed **(A)**. At 16 hr postinfection, floating cells exhibit large peripheral chromatin clumps, surface blebbing **(B)**, and cytoplasmic vacuolation and dilated nuclear pores **(C)**. In some cells more marked alterations such as derangement of chromatin structure **(D)** can be distinguished. Viral particles are also observable in the perinuclear cisternae of detached cells undergoing apoptosis **(E**, inset). At 24 hr postinfection, cells display the typical markers of apoptosis and viral particles released from disrupted and deranged cell cytoplasm are observed **(F)**. Even at this latest stage, mitochondrial integrity is still maintained (arrowhead). Nuclear segmentation seems to proceed from the third phase shown in D through E down to the complete micronuclei formation (arrows) shown in F. Bars represent 1 μm.

REFERENCES

Ameisen JC, Capron A (1991): Cell disfunction and depletion in AIDS: The programmed cell death hypothesis. Immunology Today 12:102–105.

Bertrand R, Sarang M, Jenking J, Kerrigan D, Pommier Y (1991): Differential induction of secondary DNA fragmentation by topoisomerase II inhibitors in human tumor cell lines with amplified c-mic expression. Cancer Research 51:6280–6285.

Blacklow NR, Greenberg HB (1991): Viral gastroenteritis. The New England Journal of Medicine 325:252–264.

Bursch W, Oberhammer F, Schulte-Hemann R (1992): Cell death by

apoptosis and its protective role against disease. Trends in Pharmacological Science 13:245–251.

Cohen JJ (1991): Programmed cell death in the immune system. Advances in Immunology 50:55–85.

Cohen GM, Sun XM, Snowden RT, Dinsdale D, Skilleter DN (1992): Key morphological features of apoptosis may occur in the absence of internucleosomal DNA fragmentation. Biochemical Journal 286:331–334.

Desjardins LM, MacManus JP (1995): An adherent cell model to study different stages of apoptosis. Experimental Cell Research 216:380–387.

Desmet VJ (1988): Liver lesions in hepatitis B viral infection. Yale Journal of Biology and Medicine 61:61–83.

Duke RC, Cohen JJ (1992): Morphological and biochemical assays of apoptosis. In Coligan J, Kruisbeek AM, Argulies DH, Evach EM, Strober W (eds): "Current Protocols in Immunology," vol. 1. New York: John Wiley and Sons, pp 3.17.1–3.17.16.

Esolen LM, Park SW, Hardwick JM, Griffin DE (1995): Apoptosis as a cause of death in measles virus-infected cells. Journal of Virology 69:3955–3958.

Estes MK (1996): Rotaviruses and their replication. In Fields BN, Knipe DM, Howley PM (eds): "Virology," 3rd ed., vol. 2. Philadelphia: Lippincott-Raven Publishers, pp 1625–1656.

Evans VG (1993): Multiple pathways to apoptosis. Cell Biology International 17:461–476.

Groux H, Monte D, Bourrez JM, Capron A, Ameisen JC (1991): Activation of $CD4^+$ T-lymphocytes in asymptomatic HIV infected patients induce the program action of lymphocyte death by apoptosis. Comptes Rendus de l'Academie des Sciences, Serie III, Sciences de la Vie 312:599–606.

Hinshaw VS, Olsen CW, Dybdahl-Sissoko N, Evans D (1994): Apoptosis: A mechanism of cell killing by influenza A and B viruses. Journal of Virology 68:3667–3673.

Hugin AW, Vacchio MS, Morse HC (1991): A virus encoded "superantigen" in a retrovirus-induced immunodeficiency syndrome of mice. Science 252:424–427.

Jeurissen SHM, Wagenaar F, Pol JMA, Van Der Eb AJ, Noteborn MHM (1992): Chicken anemia virus causes apoptosis of thymocytes after in vivo infection and of cell lines after in vitro infection. Journal of Virology 66:7383–7388.

Kapikian AZ, Chanock RM (1996): Rotaviruses. In Fields BN, Knipe DM, Howley PM (eds): "Virology," 3rd ed., vol. 2. Philadelphia: Lippincott-Raven Publishers, pp 1657–1708.

Kerr JFR, Bishop CJ, Searle J (1984): Apoptosis. In Anthony PP, MacSween RNM (eds): "Recent Advances in Histopathology," No. 12. Edinburgh: Churchill Livingstone, pp 1–15.

Malorni W, Rivabene R, Santini MT, Donelli G (1993): N-acetylcysteine inhibits apoptosis and decreases viral particles in HIV chronically infected U937 cells. FEBS Letters 327:75–78.

Malorni W, Rivabene R, Santini MT, Rainaldi G, Donelli G (1994): Apoptosis induced by tumor necrosis factor in HIV chronically infected U937 cells is partially prevented by N-acetylcysteine and results in a decrease of viral particle production. Redox Report 1:57–64.

Malorni W, Rivabene R, Straface E, Rainaldi G, Cossarizza A, Monti D, Salviolo S, Franceschi C (1995): 3-Aminobenzamide protects from UVB-induced apoptosis by acting on cytoskeleton and substrate adhesion. Biochemical and Biophysical Research Communications 207:715–724.

McCabe MJ, Orrenius S (1992): Deletion and depletion: The involvement of viruses and environmental factors in T-lymphocyte apoptosis. Laboratory Investigation 66:403–406.

Meyaard L, Otto SA, Jonker RR, Mijnster MJ, Keet RPM, Miedema F (1992): Programmed cell death of T cells in HIV-1 infection. Science 257:217–219.

Michelangeli F, Ruiz MC, Del Castillo JR, Ludert JE, Liprandi F (1991): Effect of rotavirus infection on intracellular calcium homeostasis in cultured cells. Virology 181:520–527.

Michelangeli F, Liprandi F, Chemello ME, Ciarlet M, Ruiz MC (1995): Selective depletion of stored calcium by thapsigargin blocks rotavirus maturation but not the cytopathic effect. Journal of Virology 69:3838–3847.

Morey AL, Ferguson DJP, Fleming KA (1993): Ultrastructural features of fetal erythroid precursors infected with parvovirus B19 in vitro: Evidence of cell death by apoptosis. Journal of Pathology 169:213–220.

Nicotera P, Bellomo G, Orrenius S (1992): Calcium-mediated mechanism in chemically induced cell death. Annual Review of Pharmacology and Toxicology 32:449–470.

Oberhammer F, Wilson JW, Dive C, Morris ID, Hickman JA, Wakeling AE, Walker PR, Sikorska M (1993): Apoptotic death in epithelial cells: Cleavage of DNA to 300 and/or 50 kb fragments prior to or in the absence of internucleosomal fragmentation. The EMBO Journal 12:3679–3684.

Offit PA, Dudzik KI (1989): Noninfectious rotavirus (strain RRV) induces an immune response in mice which protects against rotavirus challenge. Journal of Clinical Microbiology 27:885–888.

Quaroni A (1985): Development of fetal rat intestine in organ and monolayer culture. Journal of Clinical Microbiology 17:352–356.

Quaroni A, May RJ (1980): Establishment and characterization of intestinal epithelial cell culture. In Harris GC, Trump BF, Stoner GD (eds): "Methods in Cell Biology," vol. 21B. New York: Academic Press, pp 403–427.

Rojko JL, Fulton RM, Renanza LJ, Williams LL, Copelan E, Cheney CM, Reichel GS, Neil JC, Mathes LE, Fisher TG, Cloyd MW (1992): Lymphocytotoxic strains of feline leukemia virus induce apoptosis in feline T4-thymic lymphoma cells. Laboratory Investigation 66:418–426.

Rouslahti E, Reed JC (1994): Anchorage dependence, integrins, and apoptosis. Cell 77:477–478.

Rousset M (1986): The human colon carcinoma cell lines HT-29 and Caco-2: Two in vitro models for the study of intestinal differentiation. Biochimie 68:1035–1040.

Schwartzman RA, Cidlowski JA (1993): Apoptosis: The biochemistry and molecular biology of programmed cell death. Endocrinology Reviews 14:133–151.

Shahrabadi MS, Babiuk LA, Lee PWK (1987): Further analysis of the role of calcium in rotavirus morphogenesis. Virology 158:103–111.

Smith CA, Williams GT, Kingston R, Jenkinson EJ, Owen JJT (1989): Antibodies to CD3/T-cell receptor complex induce death by apoptosis in immature T cells in thymic cultures. Nature 337:181–184.

Superti F, Tinari A, Baldassarri L, Donelli G (1991): HT-29 cells: A new substrate for rotavirus growth. Archives of Virology 116:159–173.

Thompson CB (1995): Apoptosis in the pathogenesis and treatment of disease. Science 267:1456–1462.

Tian P, Estes MK, Hu Y, Ball JM, Zeng CQY, Schilling WP (1995): Rotavirus nonstructural glycoprotein NSP4 mobilizes Ca^{++} from the endoplasmic reticulum. Journal of Virology 69:5763–5772.

Tropea F, Troiano L, Monti D, Lovato E, Malorni W, Rainaldi G, Mattana P, Viscomi G, Portolani M, Carmelli C, Cossarizza A, Franceschi C (1995): Virus-induced apoptosis in human peripheral blood mononuclear cells: Studies with Sendai virus and herpes virus type 1. Experimental Cell Research 217:63–70.

Vasconcelos AC, Lam KM (1994): Apoptosis induced by infectious bursal disease virus. Journal of General Virology 75:1803–1806.

Wyllie AH (1981): Cell death: A new classification separating apoptosis from necrosis. In Bowen ID, Lockshin RA (eds): "Cell Death in Biology and Pathology." London: Chapman and Hall, pp 9–34.

Wyllie AH, Morris RG, Smith AL, Dunlop D (1984): Chromatin cleavage in apoptosis: Association with condensed chromatin morphology and dependence on macromolecular synthesis. Journal of Pathology 142:67–77.

Yamada T, Yamaoka S, Goto T, Nakai M, Tsujimoto Y, Hatanaka M (1994): The human T-cell leukemia virus type 1 Tax protein induces apoptosis which is blocked by the Bcl-2 protein. Journal of Virology 68:3374–3379.

Zhivotovsky B, Cedervall B, Jiang S, Nicotera P, Orrenius S (1994): Involvement of Ca^{2+} in the formation of high molecular weight DNA fragments in thymocyte apoptosis. Biochemical and Biophysical Research Communications 202:120–127.

Zychlinsky A (1993): Programmed cell death in infectious diseases. Trends in Microbiology 1:114–117.